



Nattectin a fish C-type lectin drives Th1 responses *in vivo*: Licenses macrophages to differentiate into cells exhibiting typical DC function

Tania Cristina Saraiva^a, Lidiane Zito Grund^a, Evilin Naname Komegae^a, Anderson Daniel Ramos^a, Katia Conceição^a, Noemia M. Orri^b, Monica Lopes-Ferreira^a, Carla Lima^{a,*}

^a Immunoregulation Unit, Special Laboratory of Applied Toxinology (CEPID/FAPESP), Butantan Institute, São Paulo, Brazil

^b Medical Investigation Laboratory Unit 56, School of Tropical Medicine, University of São Paulo, São Paulo, Brazil

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ABSTRACT

Considerable efforts are currently focused on the biology of DC in view of their possible clinical use as adjuvant for the generation of antigen-specific immunity and lifelong immunologic memory or for the treatment of tumors. We assessed the role of Nattectin a C-type lectin identified in the *Thalassophryne nattereri* fish venom in DC maturation. Nattectin induced a significant neutrophilic recruitment into peritoneal cavity of mice, followed by macrophages, with lipidic mediators and IL-12 p70 synthesis. Macrophages derived from 7 day-Nattectin mice were CD11c + CD11b^{low}Ly6^{high}F4/80^{high} and express high levels of MHC class II and CD80 molecules. Culture of peritoneal exudates derived macrophages from 7 day Nattectin-mice and immature BMDCs with Nattectin markedly increased the surface expression of CD40, CD80, CD86, and MHC class II in a dose-dependent manner, and the production of MMP-2 and MMP-9 distributed in nucleus and cytoplasm of cells, that was associated with strong activity in the culture supernatant. Nattectin treated DCs secreted IL-12 p70 and IL-10. The Nattectin-treated BMDC or macrophage-derived DCs were highly efficient at Ag capture. The specific immune response elicited by Nattectin was characterized by the production of specific antibodies IgG1 and mainly IgG2a with IL-10 and IFN- γ synthesis by splenic cells. These results enable us to address that Nattectin induces the recruitment of Ly6C^{high} monocytes into the peritoneum, which exhibit a pro-inflammatory profile, where they differentiate into proliferating F4/80^{high} macrophages. Macrophage-derived DCs mature in the presence of the cytokine milieu generated against Nattectin, exhibiting T cell co-stimulatory molecule expression and induced a Th1 polarized response.

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1. Introduction

Dendritic cells (DCs) represent an extremely plastic and versatile cell type, which play a crucial role not only in the innate immunity and initiation and control of adaptive immunity, but can also contribute to the induction of tolerance. Immature DCs monitor the periphery, capturing and processing of antigens (Ags). Monocytes represent an abundant and heterogeneous population of circulating cells that can be rapidly recruited to sites of inflammation and immune response where they differentiate into DCs. Upon receiving danger signals such as the presence of inflammatory cytokines, (IL-1, TNF- α) DCs undergo phenotypic changes and migrate via lymphatics to local lymph nodes. Immunogenic DCs present Ags in the context of MHC along with co-stimulatory receptors and initiate T cell responses [1], including rapidly polarizing the immune response to either Th1 or Th2

types [2] and improving T cell memory [3]. The migration of immunogenic DCs into secondary lymphoid tissues requires three major classes of molecules: chemokines [4], which direct DCs to target sites; adhesion molecules [5]; and matrix metalloproteinases (MMP) involved in the degradation of the extracellular matrices (ECM), as MMP-2 and MMP-9 [6,7].

Lectins are widely distributed from *Caenorhabditis elegans* to *Homo sapiens*, that bind specifically and reversibly to carbohydrate structures, particularly the sugar moiety of glycoconjugates and glycoproteins. C-type lectins are proteins that contain a carbohydrate recognition domain (CRDs) and bind carbohydrate structures in a Ca²⁺-dependent manner. Calcium ions are directly involved in ligand binding, as well as in maintaining the structural integrity of the CRD that is necessary for the lectin activity, but many C-type lectins are Ca²⁺-independent and may not necessarily bind to sugar ligands [8]. The specificity of C-type lectins can be generally divided into two categories: a) the mannose (Man)-specific C-type lectins, such as DC-SIGN, that possesses specificity for Man and/or fucose (fuc) terminated glycans, b) and galactose- (Gal) specific C-type lectins, such as macrophage galactose lectin, that recognize Gal- and N-acetylgalactosamine (GalNAc) terminated glycans [9].

* Corresponding author at: Special Laboratory of Applied Toxinology, Butantan Institute, Av. Vital Brazil, 1500, Butantan 05503-009, São Paulo, Brazil. Tel.: +55 11 3726-1024; fax: +55 11 3676 1392.

E-mail address: carlalima@butantan.gov.br (C. Lima).

Since the identification of Electrolectin, the first galectin-specific lectin isolated from the electric organs of the eel *Electrophorus electricus* [10], a substantial number of lectins have been identified in other fish [11]. These lectins are distributed in the epidermis and also in the epithelia of the upper digestive tract and gills, fluids and cells where they exhibit agglutinating or opsonizing activity against the fish pathogen *Vibrio anguillarum* [12,13]. The Indian catfish (*Clarias batrachus*) expresses a Ca^{2+} -dependent galactose-specific serum lectin that significantly alters the viability and pathogenicity of various strains of the bacteria *Aeromonas* and produces a dose-dependent increase in bactericidal activity of fish macrophages [14].

Fish lectins have been extensively described within tissues and cells associated with host defensive functions (branchial epithelium, renal interstitium, hepatic sinusoidal, intestinal sub-mucosal granular layer, skin and particularly within circulating granulocytes), however only recently, a tetrameric lectin belonging to the family of mannose-binding B-type was isolated from the mucus and sting of *Scorpaena plumieri* venomous fish [15].

Recently, a C-type lectin Nattectin was identified in the venom of the Brazilian venomous fish *Thalassophryne nattereri*, through cDNA library, and by isolation with cation-exchange chromatography [16]. Nattectin revealed a single component with a pI of 9.73 and molecular mass of 15 kDa, containing six conserved cysteine residues, with one CRD (QPD). The binding specificity of Nattectin for glycoproteins was revealed to be Gal- β [1–3]-N-acetylgalactosamine, and Ca^{2+} is indispensable for expressing their hemagglutination activity. We also demonstrated that Nattectin induces neutrophil accumulation into the peritoneum, demonstrating that it plays an important role in defense systems against pathogens and parasites [17].

Considerable research efforts are currently focused on the biology of DC in view of their possible clinical use as immune adjuvants for the generation of antigen-specific immunity and lifelong immunologic memory or for the treatment of chronic infectious diseases and tumors [18–21]. Recently, Dai and coworkers [22] have shown that Galectin-9 induces human DC maturation *in vitro*, as revealed by up-regulation of the surface expression of co-stimulatory and HLA molecules and of IL-12 production in a dose-dependent manner, and that DCs treated with Gal-9 promote both T cell proliferation and production of Th1 cytokines, suggesting that Gal-9 plays an important role not only in innate immunity by modulating granulocyte functions but also in acquired immunity by inducing DC maturation.

Despite the importance of maturation state of dendritic cells in initiating adaptive immune responses and in DC-based vaccine therapies, the choice of reagent for DC maturation *in vitro* before administration is critical. Here we reported for the first time the effects of Nattectin, a C-type lectin from venomous fish on the phenotypic and functional maturation of murine bone marrow-derived DC (BMDC), and its *in vivo* action in the development of innate and adaptive immune responses.

2. Material and methods

2.1. Animals

Male 8- to 10-week-old BALB/c mice were purchased from the Butantan Institute (São Paulo, Brazil). Animals were housed in a laminar flow holding unit (Gelman Sciences, Sydney, Australia) in autoclaved cages on autoclaved bedding, in an air-conditioned room in a 12-hour light/dark cycle. Irradiated food and acidified water were provided *ad libitum*. All the procedures involving animals were in accordance with the guidelines provided by the Animal Ethics committee of the Butantan Institute, Brazil.

2.2. Reagents and Abs

Nattectin obtained by HPLC from *T. nattereri* venom was confirmed by MALDI-TOF mass spectrometry (Amersham Biosciences) according

to [17]. Endotoxin Detoxi-Gel™ (Pierce Chemical Co.) was used according to the manufacturer's instructions to remove >99% of the contaminating LPS in the Nattectin solution (resulting in a total dose <0.8 pg LPS), which was measured by Limulus Amoebocyte Lysate (BioWhittaker). Recombinant mouse (rm) GM-CSF was purchased from R&D Systems. ELISA kits for murine cytokines and chemokines or lipidic mediators were purchased from BD Pharmingen or Cayman Chemical, respectively. FITC-, PE- or PECy5-conjugated mAbs used to detect the expression of CD11b (Mac-1 α chain), CD11c (integrin α x), CD4 (L3T4), CD8a (Ly2), CD45R/B220 (RA3-6B2), CD40 (3/23), CD80 (16-10A1), CD86 (GL1), IA/IE (M5/114.15.2), purified F4/80R (6 F12), and Ly-6C (AL-21) by flow cytometry, as well as isotype-matched control mAbs, were purchased from BD Pharmingen. To detect MMP localization, anti-MMP-2 and anti-MMP-9 from R&D Systems were purchased (909-MM and 924-MP).

2.3. Nattectin-induced peritonitis

Different groups of mice were injected intraperitoneally (i.p.) with 10 μg of Nattectin diluted in 500 μl of sterile saline. Mice only injected with saline were used as control. At time points indicated (1, 6, 24 h) after injection, animals were killed by CO_2 asphyxiation, peritoneal cells were recovered by peritoneal lavage using 5 ml of ice-cold, sterile PBS plus 0.1% EDTA. After lyses in Tris-buffered ammonium chloride (pH 7.2) buffer and centrifugation, the supernatant from cell suspension was collected for cytokine and chemokine analyses, and the cell pellet was resuspended for leukocyte cell counts. Using a hemocytometer and cytocentrifuge, slides were prepared, air dried, fixed in methanol, and stained (Wright-Giemsa, Scientific Products, Chicago, IL). For differential cell counts, 300 leukocytes were enumerated and identified as mononuclear cells or polymorphonuclear neutrophils, on the basis of staining and morphologic characteristics using Axio Imager A1 (Carl Zeiss, Germany).

2.4. Modulation of peritoneal macrophages by Nattectin

Peritoneal cells ($5 \times 10^5/\text{ml}$) from 24 h or 7 day-Nattectin exudates were seeded to plate substratum at 37 °C for 18 h. Non-adherent cells were removed by washing with warm PBS and adhered macrophages were *in vitro* re-stimulated with Nattectin (1 $\mu\text{g}/\text{ml}$) or medium alone. After 4 h, the supernatants were stored for cytokines determination, and adhered macrophages were washed, counted, and resuspended in FACS buffer (1% BSA in PBS containing 0.01% NaN_3). For phenotypic analysis, cells ($0.2\text{--}1 \times 10^6$ cells/stain) were initially incubated with either 10% mouse serum or CD16/CD32 (Fc block) for 20 min at 4 °C. Subsequently, cells were incubated with FITC-, PE- or PECy5-conjugated mAbs for CD11b (Mac-1 α chain), CD11c (integrin α x), CD40 (3/23), CD80 (16-10A1), CD86 (GL1), IA/IE (M5/114.15.2), purified F4/80R (6 F12), and Ly-6C (AL-21). All incubations were performed on ice for 20 min and were followed by three washes with FACS buffer. Appropriate isotype controls were used in all cases. For flow cytometric analysis, a typical forward and side scatter gate was set to exclude dead cells and aggregates; a total of 10^4 events in the gate were analyzed using a FACScalibur and Cell Quest Pro software (BD Biosciences, San Jose, CA).

2.5. Generation of bone marrow derived DC

DCs were generated from murine bone marrow cells, as described by Inaba et al. [23]. Briefly, BM was flushed from the tibiae and femurs of naïve or Nattectin-injected BALB/c mice and depleted of red cells with ammonium chloride. The cells were plated in culture plates (0.2×10^6 cells/ml; 8 ml/plate) in RPMI 42401 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 0.1% 2-ME, 0.05 $\mu\text{g}/\text{ml}$ asparagin/vitamin, 10 mM HEPES (pH 7.4), 20 ng/ml rmGM-CSF at 37 °C, 5% CO_2 .

On day 3 of the culture, floating cells were gently removed, and fresh medium was added. On day 6 and 8, half of fresh medium was added to the culture. On day 10, non-adherent cells and loosely adherent proliferating DC aggregates were harvested for analysis or stimulation. On the last day of culture, 94% or 71% more of the non-adherent cells expressed CD11b or CD11c, respectively (data not shown).

2.6. Stimulation of immature DC by Nattectin

Nattectin (0.1, 1, and 10 $\mu\text{g/ml}$) or sterile PBS alone was added to cultures of isolated immature DC in six-well plates (10^6 cells/ml; 3 ml/well). Following treatment with increasing concentrations of Nattectin for 24 h, DC viability was analyzed using an annexin V binding assay (BD Biosciences) according to the manufacturer's guidelines. We tested whether Nattectin activity is mediated by their carbohydrate-binding activity; therefore, different concentrations of lactose (10 or 30 mM) were added to the culture medium, with sucrose at the same concentration serving as a control. The medium used in the culture, to stimulate DC with Nattectin, was supplemented with GM-CSF, because the ability of DC to capture Ag is lost if DC are cultured without GM-CSF.

2.7. Effect of Nattectin on endocytosis of peritoneal macrophages and BMDC

Peritoneal-derived macrophages or BMDC from control- or Nattectin-mice obtained after 7 day pos-injection were washed, resuspended in complete medium, and treated with 1 $\mu\text{g/ml}$ of Nattectin at 4 °C for 4 h. After washing, cells ($1 \times 10^6/\text{ml}$) were then incubated at 37 °C with 0.1 mg/ml of FITC-dextran (0.431- μm , Sigma–Aldrich, St Louis, MO, USA) for 90 min. Uptake was stopped by adding cold PBS containing 2% FCS and 0.01% NaN_3 . Cells were then washed four times and analyzed by flow cytometry (FACSCalibur, BD-Pharmingen, San Diego, CA, USA). Results of endocytosis of FITC-dextran were expressed as mean fluorescence intensity.

2.8. Flow cytometric analysis

Cells (10^6) were incubated in staining buffer (PBS with 2% FBS and 0.1% sodium azide) containing anti-CD16/CD32 Ab (Fc γ III/II, BD Pharmingen, San Diego, CA) for 30 min on ice to block the nonspecific binding of Igs. Cells were stained with fluorochrome-conjugated mAb at a concentration of 0.5 $\mu\text{g}/10^6$. Cells stained with the appropriate isotype-matched Ig were used as negative controls. After staining, the cells were fixed with 2% w/v formaldehyde analyzed immediately on a FACScaliber flow cytometer using CellQuest 3.1 software (Becton Dickinson, San Jose, CA). The instrument had a standard optical filter configuration with band pass filters of 530/30-nm and 585/44-nm for FL1 (FITC-conjugated antibodies) and FL2 (PE-conjugated antibodies) data acquisition, respectively. For the analysis of forward-angle light scatter, side-angle light scatter, and cell surface receptor expression, data were acquired in real time. Data were recorded as geometric mean fluorescence intensity (MFI) and percent fluorescent-positive cells. The instrument was standardized before phenotypic analysis with Calibration beads (Fluorospheres 6-Peak; Dako Cytomation, Carpinteria, CA) and cleaned with sequential washes of distilled water, 10% vol/vol hypochlorite and distilled water before data acquisition.

2.9. Quantitation of cytokines and chemokines

Culture supernatants were analyzed by ELISA. The OD_{450} of triplicate samples was determined and corrected by a microplate reader, with readings at 570 nm. The minimum detection levels for IL-1 β , IL-6, TNF- α , and MCP-1 was 7.8 pg/ml and for KC, IL-10, IL-12 p70, and IFN- γ was 15.6 pg/ml.

2.10. Eicosanoid assays

Concentrations of LTB4 and PGE2 were measured in the peritoneal exudate lavage fluid collected 6 h after Nattectin or saline injection, by a specific enzymatic immunoassay, using a commercial kit (Cayman Chemicals, MI, USA). In brief, 100 μl aliquots of each sample were incubated with the eicosanoid conjugated with acetylcholinesterase and the specific rabbit antiserum in 96-well microtitration plates, coated with anti-rabbit IgG mouse monoclonal antibody. After addition of the substrate, the absorbances of the samples were recorded at 412 nm in a microplate reader, and concentration of the eicosanoid was estimated from standard curve.

2.11. Immunocytochemical localization of matrix metalloproteinases (MMPs)

In 8-well tissue culture coated slides, 10^6 immature DC were cultured in the presence of 10 $\mu\text{g/ml}$ of Nattectin for 24 h according to Brooks et al., [24] and Yu and Stamenkovic, [25]. After washing slides were air-dried and then fixed with 4% formaldehyde in PBS and incubated in this fixative for 20 min at RT. Samples were washed once with PBS for 5 min. Cells were permeabilized with 1% Triton X-100 in PBS for 10 min and then washed with PBS. To block nonspecific binding sites, Dako block (Life Technologies) was applied for 10 min at room temperature. Samples were incubated for 1 h with primary antibodies to MMP-2 and MMP-9 at a concentration of 20 ng/ml in Dako sample dilution buffer (Life Technologies). After several rinses with PBS, the samples were incubated with secondary antibody for 30 min (Mouse IgG RPE Goat F(Ab) $_2$; Dako). After washings and drying, slides were mounted with ProLong Antifade Kit (Molecular Probes, Eugene, OR). A number of controls were performed to verify the specificity of the antibodies that were used in this study. No signal was detected when the primary or secondary antibody was used alone (data not shown). Isotype controls were always stained in parallel. Samples were viewed with an Axio Imager A1, Carl Zeiss (Germany) microscope with 63 $\times$ and 100 $\times$ objectives (NA = 1.4). MMP-2- and 9-specific immunoreactivity was quantified in that the number of positive cells per field of vision at 100 $\times$ magnification was counted by two independent investigators (TSC and CL).

2.12. MMP activity measurement

Supernatants from immature DC cultured with Nattectin were supplemented with an equal volume of zymograph sample buffer containing 4% SDS (Bio-Rad, Hercules, CA, USA). The sample mixture was incubated for 10 min at room temperature and loaded into a 10% SDS-polyacrylamide gel containing 0.1% gelatin. Following electrophoresis, the gel was incubated in 2.5% Triton X-100 for 30 min at room temperature and then incubated in a buffer containing 50 mmol/l Tris, 0.2 mmol/l NaCl, 5 mmol/l CaCl $_2$ and 0.02% Brij-35 (pH 7.6) at 37 °C for 10 h. Subsequently, the gel was stained with 0.25% Coomassie Brilliant Blue for 3 h at room temperature and destained in a buffer containing 30% methanol and 10% glacial acetic acid for visualizing bands of enzymatic activity. Purified murine MMP-2 and MMP-9 was used as a size marker.

2.13. Generation of immune response for Nattectin

In order to characterize the immune responses to Nattectin, mice ($n = 5/\text{group}$) were intraperitoneally (i.p.) injected two times with Nattectin (10 μg at 7-day intervals) with 1.6 mg Al(OH) $_3$ as adjuvant. Control animals were immunized only with adjuvant on day 0. Fourteen days after the first immunization mice were bled and the humoral immune responses were monitored by Abs titer and cytokine production by Ag-specific splenic cells.

2.14. Titration of antibodies by ELISA

Plasma was tested for IgG1 or IgG2a antibodies using Nattectin-coated 96-well plates and biotinylated goat anti-mouse IgG1 and IgG2a antiserum. The reactions were developed with streptavidin-horseradish peroxidase complex (Sigma Chemical Co. St. Louis, Mo, USA), O-phenylenediamine (OPD) and H_2O_2 and the plates were read at 490 nm on an automated ELISA reader (Victor 3, Multilabel Counter, PerkinElmer). The results were expressed as the mean $\pm$ SEM absorbance.

2.15. Statistical analysis

All values were expressed as mean $\pm$ SEM. Parametric data were evaluated using analysis of variance, followed by the Tukey test for multiple comparisons. Non-parametric data were assessed using the Mann-Whitney test. Differences were considered statistically significant at $p < 0.05$. The SPSS statistical package (Release 8.0, Standard Version, 1997) was employed.

3. Results

3.1. Nattectin shows pro-inflammatory activity in murine model of peritonitis

Neutrophils are rapidly mobilized from the blood to the site of inflammation during acute inflammatory reactions. To determine the type of leukocytes being recruited into the peritoneal cavity of mice following Nattectin challenge, we performed cytospins and leukocyte differential counts on the peritoneal exudates. Leukocyte recruitment progressively increased into the peritoneum of mice challenged with Nattectin over the first 1 h, which was maintained at 24 h. Nattectin induced a 4.5-fold increase in neutrophil numbers, beginning at 1 h and increasing until 6 h thereafter (Fig. 1A and B). Neutrophil counts were diminished at 24 h, concomitant with a significant increase in peritoneal macrophage infiltration (Fig. 1C).

The eicosanoid mediators and inflammatory cytokines are known to function as a potent neutrophil chemoattractant. As shown in Fig. 2, peritonitis induced by Nattectin was accompanied by elevated levels of LTB4 (Fig. 2A) and PGE2 (Fig. 2B) mediators as well as IL-1 β (Fig. 2C) and IL-6 (Fig. 2D). In addition, Nattectin promoted the release of CXC and CC, chemokines controlling neutrophils and macrophage recruitment, KC (CXCL8) and MCP-1 (CCL2), respectively (Fig. 1A and 3B). Following Nattectin challenge, IL-12 p70 levels significantly increase in the peritoneal exudates at 24 h (Fig. 3C) and after 6 h, a sustained release of IL-10 was observed (Fig. 3D). Nattectin did not induce detectable amounts of TNF- α or NO in peritoneal exudates (data not shown).

3.2. Nattectin induces the differentiation of macrophage into cells exhibiting typical DC function

To further characterize peritoneal-derived macrophages in Nattectin-mice, we examined the expression of macrophage/DC markers, costimulatory molecules and the production of cytokines. As expected, peritoneal-derived macrophages from control exudates were CD11c⁺ and express high levels of CD11b, F4/80R, and Ly6C (control bars, Fig. 4A). Peritoneal-derived macrophages from 24 h-Nattectin exudates were CD11c⁺ and express low levels of CD11b, and were negative for Ly6C or F4/80R (gray bars, Fig. 4A). Peritoneal-derived CD11c⁺ CD11b^{low} macrophages from 7 day-Nattectin exudates have intensified expression of Ly6C (167-fold) and F4/80R (161-fold) (black bars, Fig. 4A).

Low levels of CD80, CD86, and MHC class II were observed in peritoneal-derived macrophages from control- or 24 h-Nattectin exudates (Fig. 4B, control and gray bars, respectively). Peritoneal-

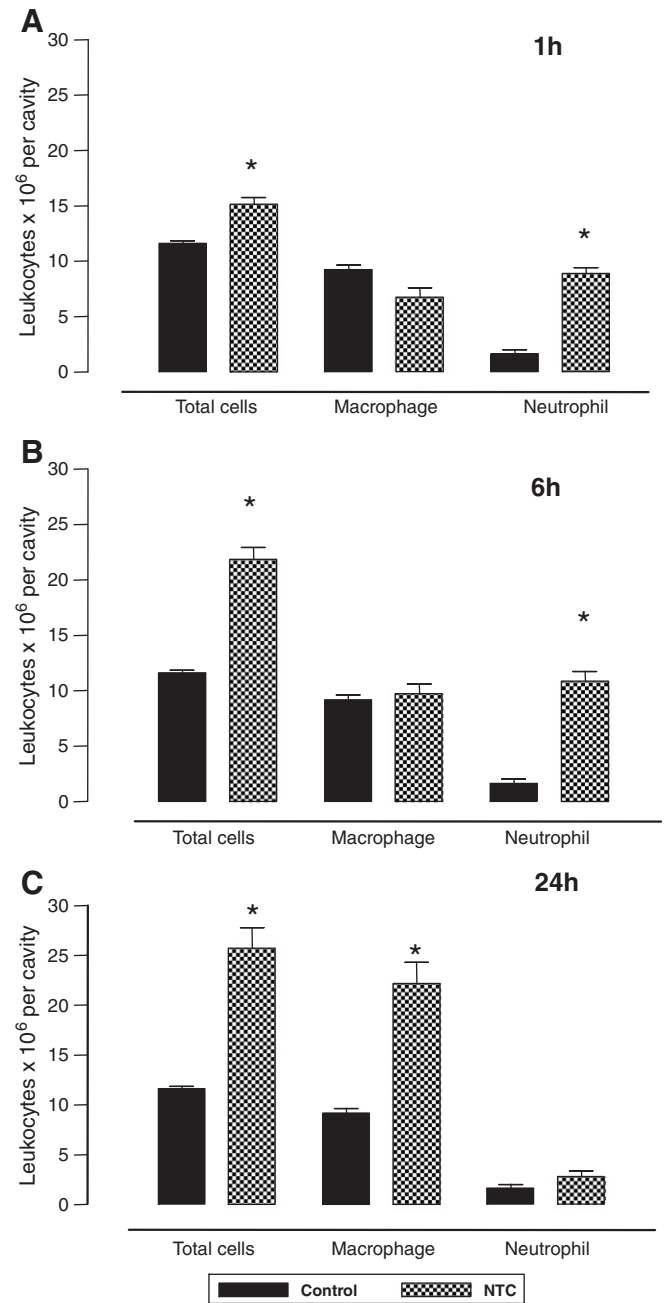


Fig. 1. Nattectin shows pro-inflammatory activity in a murine model of peritonitis. At different time points (1 h – A, 6 h – B and 24 h – C) after i.p. injection of 10 μ g of the Nattectin animals were killed and peritoneal cavities were washed for total, neutrophils, and macrophage cell count. Mice only injected with saline were considered as control. The results represent the mean $\pm$ SEM. * $p < 0.05$ compared with control-group.

derived macrophages from 7 day-Nattectin exudates expressed high levels of CD80 and mainly MHC class II (Fig. 4B, black bars). Thus, macrophages derived from 7 day-Nattectin mice were CD11c⁺ CD11b^{low} Ly6^{high} F4/80R^{high} and express high levels of MHC class II and CD80 molecules.

Of interest, peritoneal-derived macrophages from 24 h-Nattectin exudates stimulated *in vitro* with medium alone produced IL-6 and IL-12 p70 cytokines (Fig. 5A and 5B). The level of IL-12 p70 in peritoneal-derived macrophages from 24 h-Nattectin exudates remained high after re-stimulation with Nattectin, and also peritoneal-derived macrophages from 7 day-Nattectin exudates induced bioactive IL-12 p70 cytokine production after re-stimulation with Nattectin (Fig. 5B).

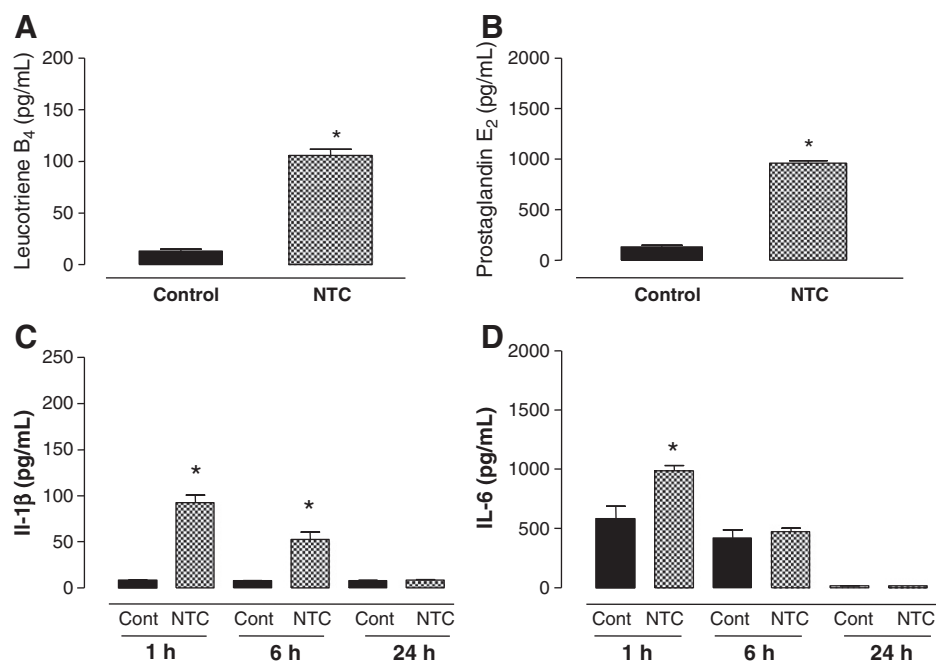


Fig. 2. Inflammatory mediators produced in response to Nattectin. LTB₄ (A), PGE₂ (B), IL-1 β (C) and IL-6 (D) were analyzed by ELISA in the peritoneal exudates of Nattectin-mice 1, 6 or 24 h after i.p. injection. The results represent the mean $\pm$ SEM. * $p < 0.05$ compared with control-group.

TNF- α or IL-10 was not detected in supernatant of peritoneal derived-macrophages from control, 24 h- or 7 day-Nattectin exudates (data not shown).

Dextran is a representative Ag that is taken up by DCs primarily via specific mannose receptor-mediated endocytosis with a minor component of uptake due to constitutive fluid phase pinocytosis [26].

Here we determined whether peritoneal-derived macrophages had augmented endocytic activity after incubation with FITC-dextran particles. As depicted in Fig. 5C, Nattectin increased the endocytic capacity of peritoneal-derived macrophages from 7 day-Nattectin exudates (1.3-fold), consistent with an immunogenic APC functional phenotype.

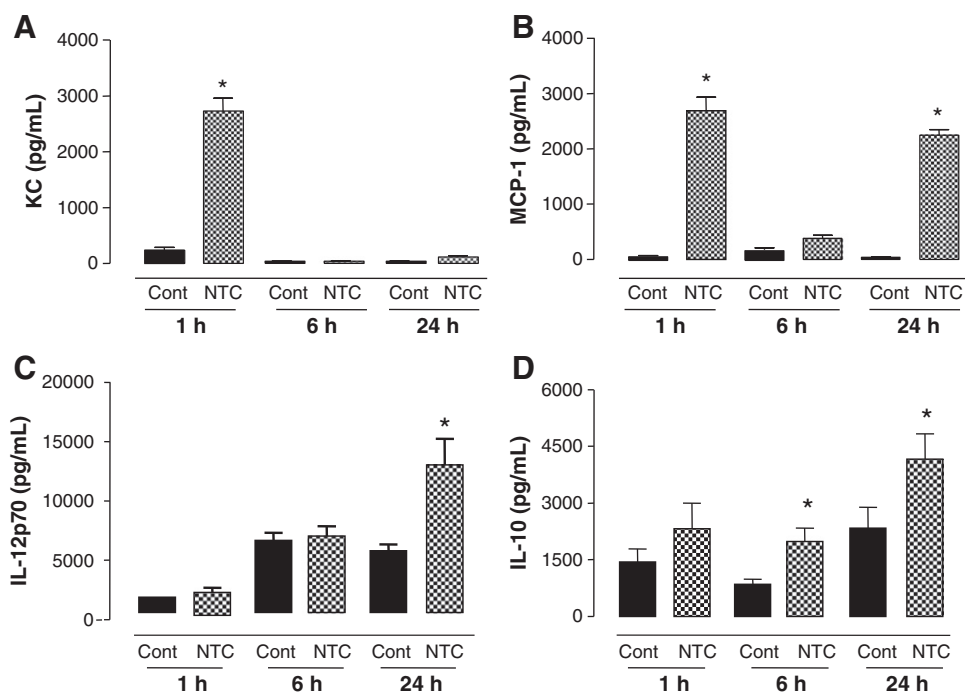


Fig. 3. Chemokines and immunomodulators induced by Nattectin. At different time points (1 h, 6 h and 24 h) after i.p. injection of 10 μ g of the Nattectin animals were killed and peritoneal exudates were analyzed for KC (A), MCP-1 (B), IL-12 p70 (C) and IL-10 (D) by specific ELISA. The results represent the mean $\pm$ SEM. * $p < 0.05$ compared with control-group.

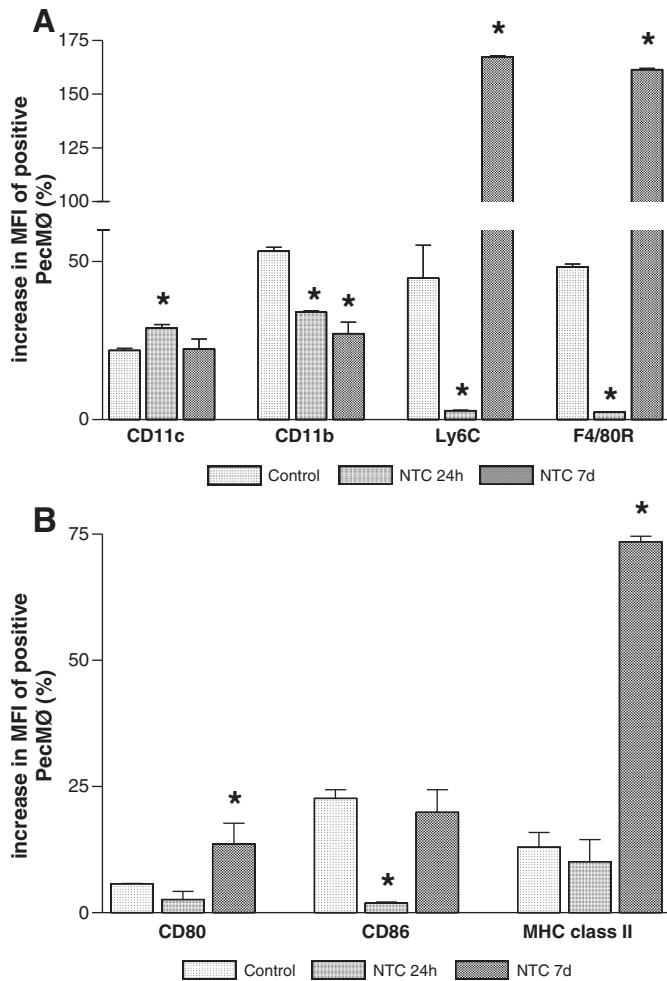


Fig. 4. Increase of co-stimulatory molecules on macrophage-derived DC by Nattectin. Peritoneal macrophages from control, 24 h or 7 day-Nattectin exudates were *in vitro* re-stimulated with Nattectin (1 μ g/ml) or medium alone. After 4 h, the geometric mean fluorescence intensity (MFI) of co-stimulatory molecules was analyzed using a FACSCalibur cytometer (BD Biosciences) and CellQuest data acquisition and analysis software.

3.3. *In vivo* effects of Nattectin on DC maturation

To clarify whether Nattectin had *in vivo* effects on BM cell differentiation into DCs, mice were killed 7 days after injection of saline or Nattectin for BMDC culture. Expansion of BMDC from femur of 7 day-Nattectin mice in the presence of GM-CSF throughout a period of 10 days led to a $2.7 (\pm 0.4)$ -fold increase in total cell number compared to BMDC recovered in control-mice (Fig. 6A). In Fig. 6B, CD11c + MHC class II + BMDC from Nattectin-mice expressed significantly more CD40, CD80, and CD86 than BMDC from control-mice.

3.4. *In vitro* effects of Nattectin on DC maturation

We next addressed the question of whether Nattectin acts on DC maturation in *in vitro* assay. The bone marrow cells from naïve mice incubated with GM-CSF exhibiting a typical phenotype of immature DCs (CD11b^{high}CD11c + CD8 α ^{neg} B220^{low}) with moderate levels of MHC class II molecules, but low expression of CD40, CD86, and CD80 as expected (Fig. 7A).

The abundant expression of CD40, CD80, CD86, and MHC class II molecules resembles the phenotype of mature DC, originally defined as cells that have an increased capacity to stimulate T cell proliferation. After exposure to different concentrations of Nattectin for

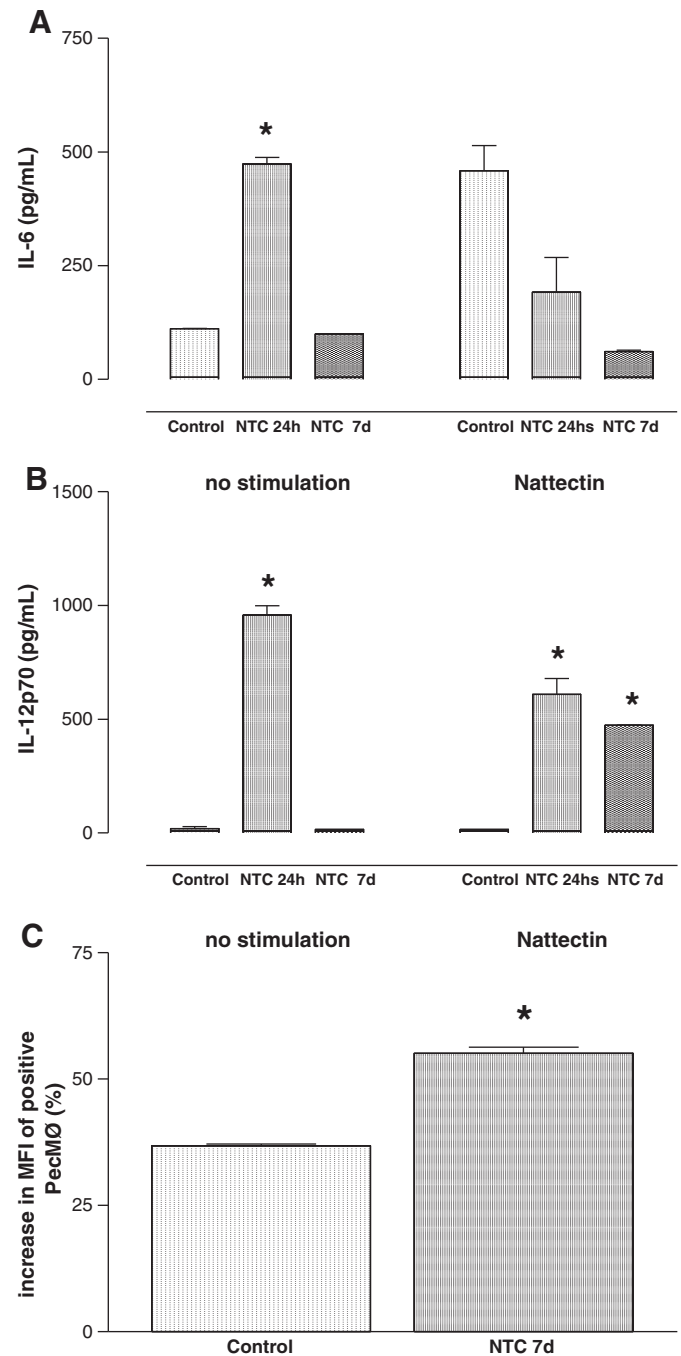


Fig. 5. Stimulatory effect of Nattectin on macrophage-derived DC. Peritoneal macrophages from control, 24 h or 7 day-Nattectin exudates were *in vitro* re-stimulated with Nattectin (1 μ g/ml) or medium alone. After 4 h, the supernatants were used to cytokines determination by ELISA (IL-6, A; IL-12 p 70, B) and endocytic capacity (C) of cells was determined by flow cytometry.

24 h, the cells acquired a mature phenotype, dose dependently up-regulating the expression of co-stimulatory molecules, mainly at 10 μ g/ml (Fig. 7A). We monitored herein DC survival on exposure to Nattectin. We found that all concentrations (0.1, 1, and 10 μ g/ml) capable of modulating DC phenotype failed to affect DC viability (data not shown).

IL-12 production by DCs is strongly linked to maturation state. In addition, Nattectin greatly increased the production of IL-12 p70 (Fig. 7B) and also induced a significant increase of IL-10 secretion (Fig. 7C). The up-regulation of the expression of co-stimulatory molecules or cytokine production by Nattectin were not suppressed

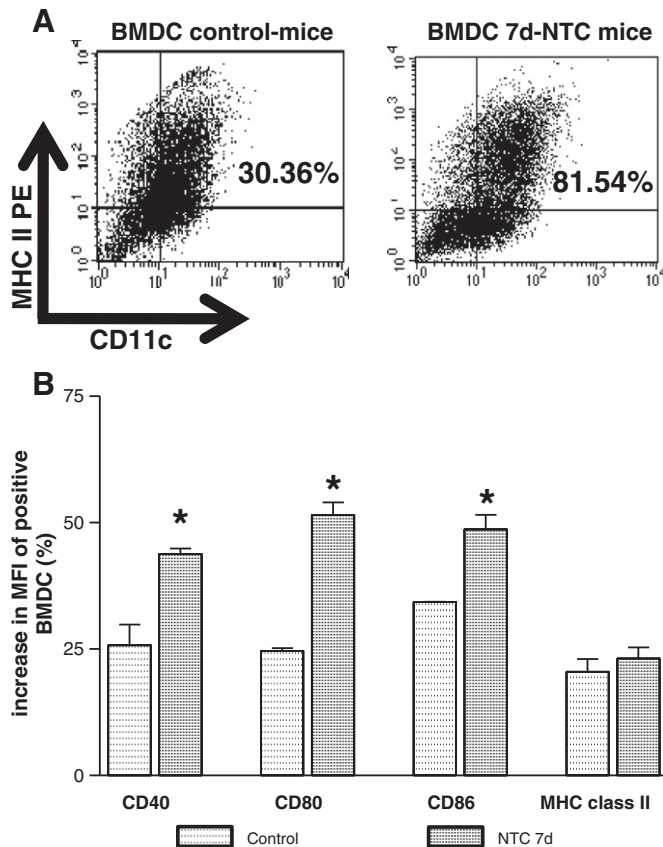


Fig. 6. Stimulatory effect of Nattectin on *in vivo* DC maturation. Mice were killed 7 days after injection of saline or Nattectin for BMDC culture in the presence of GM-CSF throughout a period of 10 days. Numbers reflect the percent of double positive cells CD11c×MHC II DC from control or NTC-mice (A). Surface expression levels of MHC class II, CD40, CD80, and CD86 were measured by the analysis of the geometric mean fluorescence intensity (MFI) using a FACSCalibur (B). Data from one experiment are representative of four independent experiments.

by lactose even at 30 mM or by polymyxin B treatment (data not shown), confirming that the β -galactoside-binding activity of Nattectin is not essential for this lectin to induce DC maturation. Since polymyxin B, an LPS inhibitor, showed no inhibitory effect on Nattectin-induced cytokine secretion, it seems likely that the activity of Nattectin is derived from its self-function rather than due to trace amounts of contamination of endotoxin.

The expression of surface molecules and IL-12 p70 secretion indicated that exposure to Nattectin significantly induced the phenotypic maturation of *in vivo* (Fig. 5C) and *in vitro* generated DC (Fig. 7B). Then, BMDC from naïve mice exhibited high capacity to take up FITC-dextran after Nattectin stimulation compared to unstimulated BMDC (3.4 ± 0.2 -fold, Fig. 7D).

After antigen acquisition, DCs undergo phenotypic changes and migrate via lymphatics to local lymph nodes. To accomplish this requires not only that DC follow chemoattractant gradients, but also that they produce MMPs enabling them to traverse the extracellular matrix. Immunocytochemistry analysis demonstrated a strong staining of MMP-9 into the cytoplasm and on the surface of Nattectin-DCs (Fig. 8D), and some DCs expressed MMP-2 (Fig. 8B). This induction of MMP-9 by Nattectin was associated with strong activity (83 kDa) in the culture supernatant of BMDC (right, Fig. 8E).

3.5. Nattectin primes Th1 phenotype *in vivo*

Immunogenic DCs are able to drive T-cell clonal expansion and differentiation, and prime immune responses. We next determined

the nature of the cellular immune response elicited by Nattectin in mice. To this end, 14 days after the first immunization with Nattectin, spleens were removed from mice immunized twice with Nattectin plus alum adjuvant and cells were *in vitro* re-stimulated with 10 μ g of Nattectin. As seen in Fig. 9, upon *in vitro* Nattectin re-challenge, spleen lymphocytes from Nattectin-mice produced the stimulatory cytokine IFN- γ (Fig. 9A) as well as the anti-inflammatory cytokine IL-10 (Fig. 9B). In contrast, there was no production of IL-2, IL-4, IL-5 or IL-12 p70 by spleen cells after stimulation with Nattectin (data not shown).

As a further assessment of the ability of Nattectin activated DC to prime an adaptive immune response *in vivo*, we measured the antibody production in mice. Th1 cells help B cells switch to IgG2a production, whereas Th2 cells are associated with the production of IgG1. Mice immunized with Nattectin had a significantly stronger specific IgG2a response (Fig. 9C), but moderate levels of specific IgG1 (Fig. 9D), indicative of a Th1 shift.

4. Discussion

C-type lectins are a main kind of pattern recognition molecule in the innate immune system. It has become clear that by binding a variety of carbohydrate structures, particularly the sugar moiety of glycoconjugates and glycoproteins both on the cell surface and in ECM, lectins deliver signals intracellularly as well as mediate cell–cell and cell–ECM adhesion [27]. Lectins thus modulate development, inflammatory, autoimmune, and infectious diseases, and tumor metastasis. Fish have evolved natural immune modulators such as galectins and C-type lectins with widespread tissue distribution, which are able to counter the host defense against microbial pathogens.

In the study presented here, we describe the biological characterization of Nattectin from the venom of the Brazilian venomous fish *T. nattereri*. *In vivo* characterization identified that Nattectin, a galactose-specific lectin belonging to the family of C-type lectins, has potent pro-inflammatory properties, promoting leukocyte recruitment into peritoneal cavity of mice, and has been shown to be dependent on the induction of several lipidic mediators, cytokines, and chemokines, including CXCL1 (KC) and CCL2 (MCP-1), which are high-affinity ligands for CXCR1/CXCR2 and CCR2, respectively. In murine peritonitis, rapid up-regulation of KC precedes the recruitment of neutrophils into peritoneum that is followed by MCP-1 up regulation and monocyte influx, culminating in resolution of leukocyte inflammation [28,29].

Many human galectins have shown pro-inflammatory activities *in vitro* and *in vivo* [22,30,31]. Gal-8 modulates neutrophil function via interaction with integrin α M [32] and Gal-3 can function as a chemokine, inducing migration of human monocytes and macrophages [33]. Similar to chemokines, this activity is mediated through a pertussis toxin (PTX)-sensitive (G-protein-coupled) pathway and associated with a Ca^{2+} influx, and thus a specific chemokine receptor(s) may be involved. If Nattectin also drives leukocyte mobilization by directly acting on chemokine receptor expressed on the cell surface it remains to be elucidated.

Furthermore, the pro-inflammatory properties of Nattectin were reinforced by the induction of IL-12 p70 production in the peritoneal exudates. The production of the bioactive p70 form of IL-12 is tightly regulated and involves separate control of the production of the p40 and p35 components by factors such as microbial products, the CD40 ligand CD154, stimulation from activated T cells, and the appropriate cytokine milieu. Thus, it appears that pro-inflammatory mediators as IL-1 β , IL-6 and PGE2 also present in the peritoneal exudates of Nattectin-mice seem to cooperate to license macrophages to differentiate into cells exhibiting typical DC function [34–37].

Differentiation of monocytes/macrophages into dendritic cells *in vivo* has been well documented [38,39]. Our results presented here

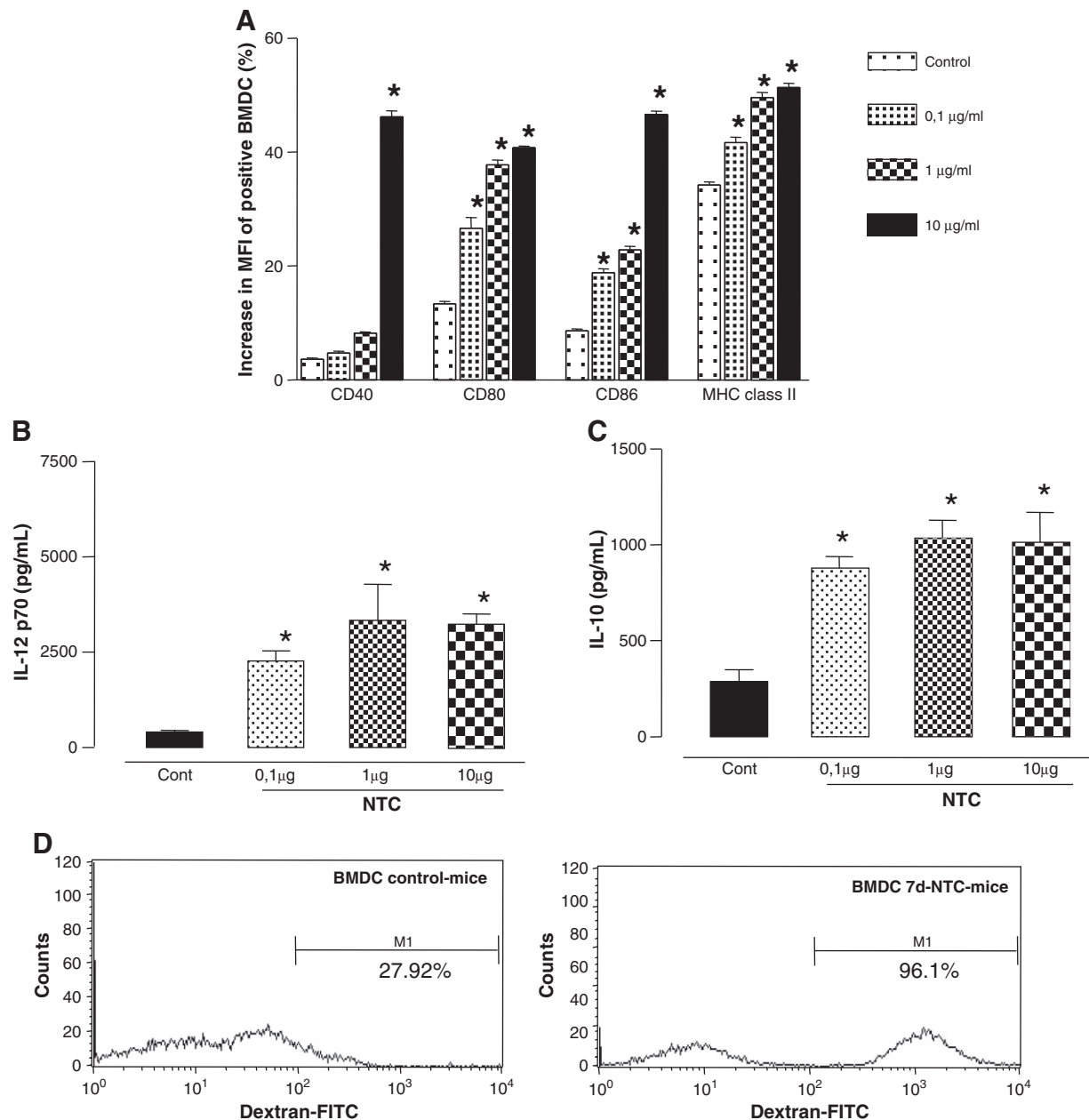


Fig. 7. Effects of Nattectin on DC phenotypic maturation. A) Immature BMDC from naïve mice were pulsed for 24 h with medium or Nattectin (0.1–10 $\mu\text{g/ml}$). Surface expression levels of MHC class II, CD40, CD80, and CD86 were measured by the analysis of the geometric mean fluorescence intensity (MFI) using a FACSCalibur (A). Culture supernatants were collected and IL-12 p70 (B) and IL-10 (C) cytokine production determined by sandwich ELISA. Endocytic capacity (D) of cells was determined by flow cytometry. Data from one experiment are representative of four independent experiments. The results represent the mean $\pm$ SEM. * $p < 0.05$ compared with control-group.

showed that macrophages mobilized to the peritoneal cavity directed by Nattectin Ag, differentiated into mature DC, as evidenced by up-regulation of Ly6C, F4/80R, CD80 and MHC class II. These macrophage-derived DCs exhibit functional attributes (augmented Ag presentation capacity with avid phagocytosis, high T cell co-stimulatory molecules expression, and enhanced capacity to activate T cells by the production of IFN- γ through the high-level of bioactive IL-12 p70 secretion), which may be important to activate T cells *in vivo*.

Subsequently we assessed the effects of Nattectin on DC functional activation using a comprehensive *in vitro* assay: BMDC generated by GM-CSF uniformly expresses high levels of CD11b^{high} CD11c + CD8 α ^{neg} B220^{low} putative of myeloid-related BMDC subset [40]. Our results showed that exposure of immature BMDC to Nattectin induced phenotypic changes characterized by enhanced expression of molecules involved in antigen presentation (MHC class II) and in co-stimulation

(CD40, CD80, CD86), concordant with conventional activation of DC by LPS or CD40L. The interaction between CD40L on CD4⁺ T cells and CD40 on dendritic cells has been shown to be indispensable for T cell activation and differentiation [41]. Thus, the up-regulation of CD40 expression and other co-stimulatory molecules in DC induced by Nattectin may contribute to significantly enhanced DC stimulatory function in driving T cells proliferation. Interestingly, these experiments clearly confirmed the stimulatory effect of Nattectin on DC maturation by demonstrating, among other events: the loading of MHC class II molecules on the cell surface of DCs, elevated IL-12 p70 production, and capacity of DCs to migrate via the lymphatics and home to the T cell-rich area of lymph nodes, through its effect on production and release of active MMP-9 [42,43].

Therefore, these results enable us to address that Nattectin induces the recruitment of Ly6C^{high} monocytes into the peritoneum, which

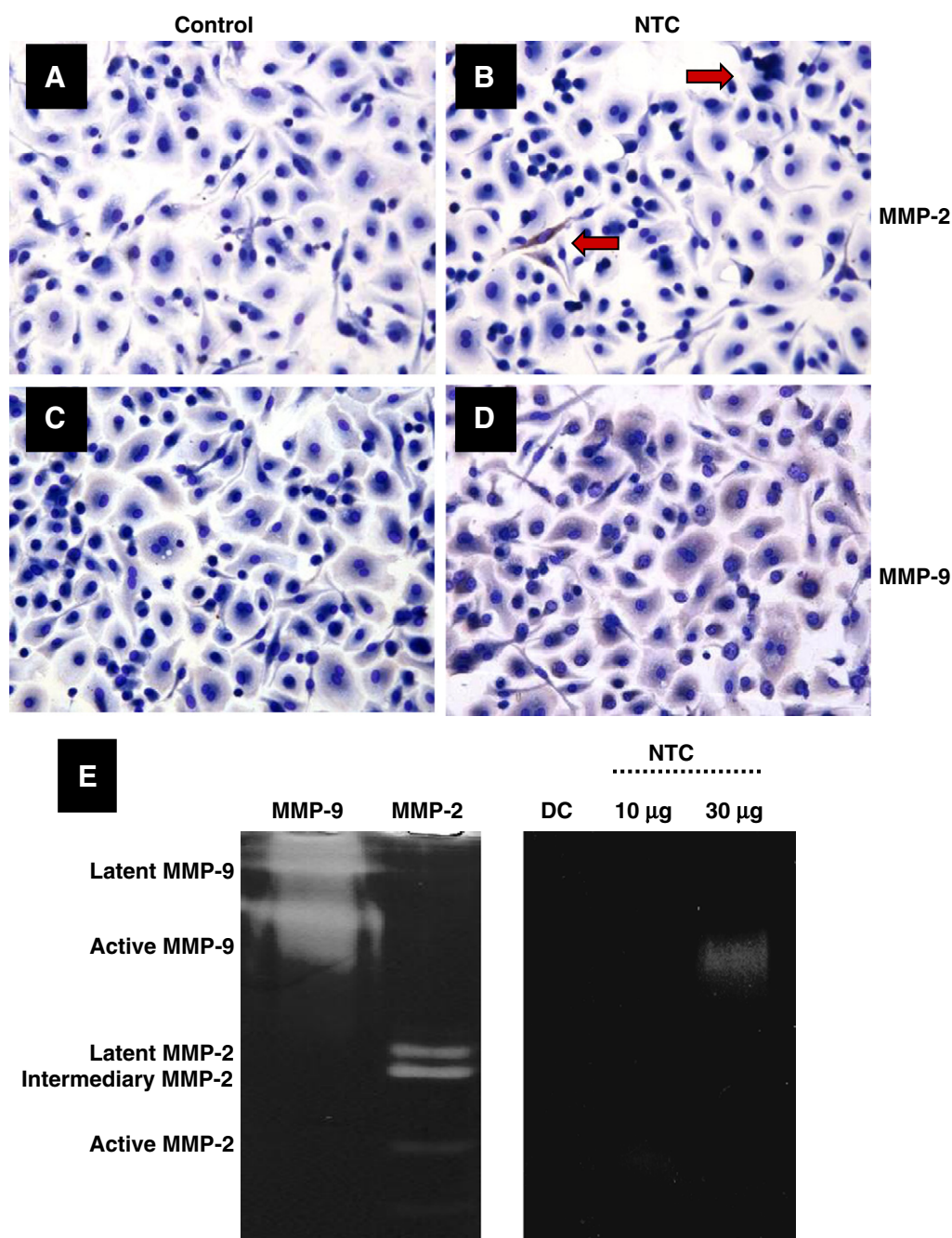


Fig. 8. Effect of Nattectin on DC production and release of active MMP-9. iDCs were pulsed for 24 h with medium only or Nattectin (10 or 30 µg/ml) and culture supernatants were collected and analyzed by MMP-2 and -9 immunohistochemical (10 µg/ml, A–D) and gelatin zymography to detect MMP-9 and MMP-2 enzymatic activity (10 or 30 µg/ml, E).

exhibit a pro-inflammatory profile, where they differentiate into proliferating F4/80R^{high} macrophages, probably positive for CCR2 and CD62L. These cells mature in the presence of the cytokine milieu generated against Nattectin, exhibiting T cell co-stimulatory molecule expression and acquire antigen and transport it to draining lymph node. In addition, our results demonstrated that in response to inflammation signals derived from Nattectin, monocytes can move quickly to sites of infection and differentiate into macrophages and DCs and bone marrow cells are also able to differentiate into mature DCs.

We also detected in a peritonitis model or *in vitro* DC maturation, both stimulated with Nattectin the production of IL-10, a cytokine that modulates both innate and adaptive immunity, primarily by exerting

anti-inflammatory effects. This finding addresses the question concerning the role of concomitant IL-10 and IL-12 p70 production induced by Nattectin. We reasoned that the failure in TNF-α and NO production by Nattectin-injected mice could be the result of anti-inflammatory effects of IL-10 [44] potentiated by regulatory effect of PGE2, such as has been proposed in models of inflammation involving the action of IL-1β and IL-10 [45,46].

Lastly, we therefore tested whether phenotypic changes induced by Nattectin supported the DC capacity to stimulate specific T cell expansion and Th1 differentiation during a close DC–T-cell interaction. Our *in vivo* results demonstrate that Nattectin induced a Th1 polarized response, characterized by high levels of specific IgG2a and Ag-specific IFN-γ-producing cells *in vivo*. These cells also did not

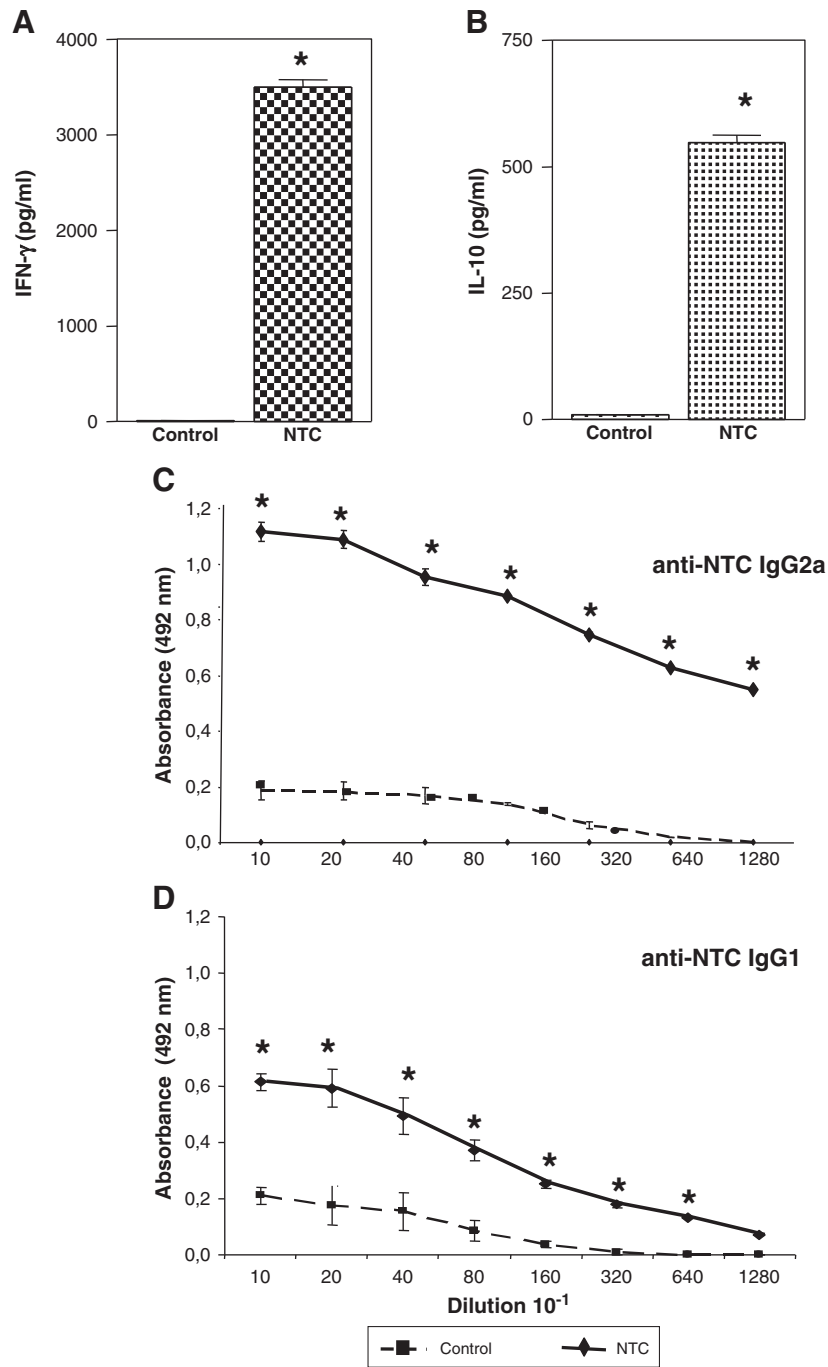


Fig. 9. Nattectin induced a Th1 polarized response *in vivo*. T cell response to Nattectin was determined from mice immunized i.p. with Nattectin (10 μ g at 7-day intervals) with 1.6 mg Al(OH)₃ as adjuvant. Control animals were immunized only with adjuvant on day 0. Fourteen days after the first immunization, mice were bled and the humoral immune responses were monitored by Abs titer (C, D) and cytokine production by Ag-specific splenic T cells (A, B). The results represent the mean $\pm$ SEM. * $p < 0.05$ compared with control-group.

produce IL-4 or IL-5, indicating that Th2 cells were not differentiated. In addition, we found that Th1 response induced by Nattectin is probably modulated by the anti-inflammatory cytokine IL-10, because the latter is produced concomitantly with IFN- γ in immunized mice.

Recently Rutz and coworkers [47] identified Notch signaling as a potent inducer of IL-10 both in developing and in already established IFN- γ -secreting Th1 cells. This effect requires co-stimulation by the cytokines IL-12 or IL-27 that depends on signal transducer and activator of transcription 4 (STAT4) signaling, confirming the role of Notch dependent Th1-derived IL-10 under inflammatory conditions in the protection of the host from Th1-induced pathology independently of

regulatory T cells [48,49]. Moreover, DCs that matured in response to Gal-9, like those treated with glucocorticoids or PGE2 [50,51] also stimulate T cells responsible for IL-10 synthesis.

Collectively, Nattectin is a novel venomous fish C-type lectin with potent pro-inflammatory capacity. This lectin acts modifying the biological responses through the control of recruitment and activation of monocytes/macrophages, licensing these cells to differentiate into cells exhibiting typical DC function. There is a general consensus that fully activated DC, expressing appropriate markers of phenotypic activation and secreting a Th1 profile of inflammatory cytokines, will be optimal for the priming of specific T cell-mediated anti-tumor

immunity by Ag-loaded DC [52]. Thus, Nattectin represent an optimal candidate adjuvant for Ag-loaded DC by its immunomodulatory activity for selecting Th1-dominant response.

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